INCREASE OF CORTICAL CALCIUM WITH AGE IN THE CELLS OF A ROTIFER, EUCHLANIS DILATATA, A PLANARIAN, PHAGOCATA SP., AND A TOAD, BUFO FOWLERI, AS SHOWN BY THE MICROINCINERATION TECHNIQUE[1]

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In the preceding study the writer (1942) demonstrated that the amount of calcium in the cortex of the leaf cells of *Elodea* increases with age. The following studies were conducted in order to discover whether a similar increase in calcium content occurs in representative animal cells.

Material and Methods

Material for analysis was chosen so as to include both multiplying and non-multiplying cells of diverse organisms. The rotifer, *Euchlanis dilatata,* and the gastrocnemius muscle of the toad, *Bufo fowleri,* were selected to provide examples of non-multiplying cells; the planarian, *Phagocata* sp., provides within the same animal both multiplying and non-multiplying cells.

Euchlanis dilatata

The stock of this rotifer was developed from a single animal found in a pond near Bloomington, Indiana. The rotifers were cultured in the laboratory on pyrex depression slides containing two drops of culture fluid (Sonneborn, 1936). A number of newly-hatched rotifers were isolated and 40 of these animals were removed and fixed for histochemical analysis. The remaining animals were cultured on the depression slides throughout the entire life span. The animals were transferred daily to fresh depressions and samples of 20 animals were taken each day for chemical analysis. The last sample was taken at the end of the fourth day at which time the rotifers manifested the characteristic

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changes of senescence. The maximum age of *Euchlanis dilatata* when grown at 30° C. is four to five days. This was determined in a number of experiments on the normal life cycle (unpublished data).

Phagocata sp.

Several hundred of the multipharyngeal planarian, *Phagocata* sp., were collected from a spring near Bloomington, Indiana, and brought into the laboratory. The planaria were examined with a dissecting microscope and measured while fully extended. The smallest animals found were 3 to 4 mm. in length; 25 of these small planaria were selected for chemical analysis. A second group of 25 animals 5 mm. in length and markedly broader than the preceding group of animals, was selected for analysis to represent the planaria of medium size, since these were most abundantly found. The largest animals were 10 mm. in length; 25 of these were selected at random for chemical analysis. On the basis of size it was assumed that the planarians 3 to 4 mm. in length were the youngest of the three groups and that the planarians measuring 10 mm. in length were the oldest. This conclusion was substantiated by a comparison of the degree of pigmentation of the animals. The smallest animals possessed the light gray pigmentation of young planaria while the large animals were a dense black, a characteristic of adult *Phagocata*.

Bufo fowleri

A collection of toads was made during the third week of August in the vicinity of Greenwood Lake, Indiana. All the animals were gathered from a relatively small area in order to reduce environmental variations to a minimum.

Six toads measuring 5.6 to 6.9 cm. in length (fully extended) were selected for analysis and pithed. The gastrocnemius muscles were carefully excised and were immediately put into absolute alcohol-formalin fixative. Six toads measuring 13.6 to 15.7 cm. were pithed and the gastrocnemius muscles excised and fixed as were the previous group. On the basis of the length measurements described above and the growth data of Hamilton (1934) whose work was done on the closely related toad, *Bufo americanus americanus* Holbrook, it was concluded that the toads measuring 5.6 to 6.9 cm. were four to five months old and that the toads measuring 13.6 to 15.7 cm. were two or more years old.

Microincineration Technique

The technique of microincineration employed in these experiments is essentially the same as that of Scott (1933a). Satisfactory results were

obtained by the author (1938) with this technique in an analysis of the localization of calcium in *Paramecium caudatum*. The experimental material was fixed in absolute alcohol-formalin (9 pts. to 1 pt.), dehydrated, cleared in xylol, and imbedded in paraffin. The rotifers and planaria were sectioned at six micra and the gastrocnemius muscle of the toad was sectioned at eight micra. The sections were mounted on clean glass slides and placed in an electric furnace. As recommended by Scott, the initial temperature increase was effected slowly, 20 minutes being required to obtain a temperature of 100° C. The temperature was then raised to and kept at 600° C. for 30 minutes which was found to be adequate for complete volatilization of the organic substances. The slides were cooled very slowly and placed without cover glasses in clean slide boxes to make possible future chemical analysis of the incinerated material. No attempt was made to analyze other elements besides calcium although some measure of the iron content of the preparations could be obtained from inspection of the untreated slides. A reddish-yellow color in the untreated incinerated preparations indicated the presence of iron and the intensity of this color could be used as an index of the iron content.

Calcium was identified chiefly by means of the alizarin reaction. (Saturated aqueous sodium alizarin sulfonate reacts with calcium to form the red precipitate of calcium alizarinate.) Occasional checks on the alizarin reactions were made by means of the delicate gypsum reaction. The reagents used in this reaction were one microdrop of 0.1 N HCl followed by one microdrop of 0.1 N H_2SO_4 (Scott). The presence of calcium was indicated by the formation of needle-like crystals of calcium sulfate. Since there was a marked diffusion of salts following the HCl treatment, precise localization of calcium could not be effected by this reaction, and for that purpose the alizarin reaction was depended upon completely. There was no piling up of the granules of ash in the incinerated sections since the material was cut at six and eight micra; that is, the incinerated preparations were but one granule deep. Consequently, an increase in the number of granules which stain red after the alizarin treatment, and thus an increase in the intensity of the staining reaction could be taken to mean that the amount of calcium had increased. Similarly, an increase in the number of calcium sulfate crystals formed, using the alternative test, was taken as an index of an increase in the calcium content.

Slides of the various organisms were stained with Ehrlich's haematoxylin, and these preparations were compared with the incinerated material for the purpose of identifying structures. As will be brought

out later in the paper, material stained with Ehrlich's haematoxylin may be used in direct determinations of the calcium content of the tissues with the intensity of staining as an index of the calcium content.

Results

Bufo fowleri

The gastrocnemius muscle of the toad when incinerated furnishes a picture very similar to that obtained by Scott (1932) in his microincineration studies on striated muscle. The sarcolemma shows in young fibers as a very fine, white line of ash. The residual ash of the sarcolemma in many preparations is so slight as to be almost invisible. The ash of the sarcolemma in old muscle fibers is strikingly different from that of young tissue. Here the line of white ash is glaring and heavy. The striations show in young and old muscle fibers as parallel rows of discrete, small white granules. However, the granules in the striations of old muscle fibers are markedly larger than those in young muscle fibers. As observed by Scott, the isotropic bands or the J bands appear to be ash free. In these preparations no trace was found of the intermediate bands of the J striation, or the Z band, although Scott reports observing the ash skeleton of the Z striation in "favorable" material.

Tests with sodium alizarin sulfonate demonstrate that the nuclei of young and old muscle fibers are both rich in calcium. The large amounts of this cation present in the nuclei rendered it impossible to estimate quantitative differences between young and old muscle fibers. The sarcolemma of young muscle fibers after alizarin treatment shows the red color characteristic of calcium alizarinate and tests with the gypsum reaction reveal the needle-like crystals of calcium sulfate. Contrary to previous experience with other tissues it was possible to use the gypsum reaction for quantitative estimations of calcium because of the large size of the muscle fibers. The sarcolemma of old muscle fibers gives the same positive tests for calcium but consistently demonstrates a marked increase in calcium content, as indicated by an increase in the staining intensity with alizarin and an increase in the number of crystals formed in the gypsum reaction. Similarly, the striations of old muscles show a marked increase in the concentration of calcium over the striations of young muscle fibers. The nuclei of both young and old muscle fibers contain varying amounts of iron as indicated by the presence of reddish-yellow granules. Iron is also present in the striations of both young and old fibers and there appears to be an increase in the iron content of the striations with age.

Phagocata sp.

Incinerated preparations of this multipharyngeal planarian show an interesting variation in the total ash content of the various tissues. The epithelial lining of the body is richest in inorganics. The pharynges show a markedly low total inorganic content in contrast to the high salt content of the epithelium (Fig. 1). The ash in the pharynges is a dull bluish-white and is uniformly scant in the various tissue layers of these organs. However, the cell membranes of the cells lining the lumina of the pharynges contain considerably more ash than other regions of these organs. Throughout all the tissues and organs that were examined

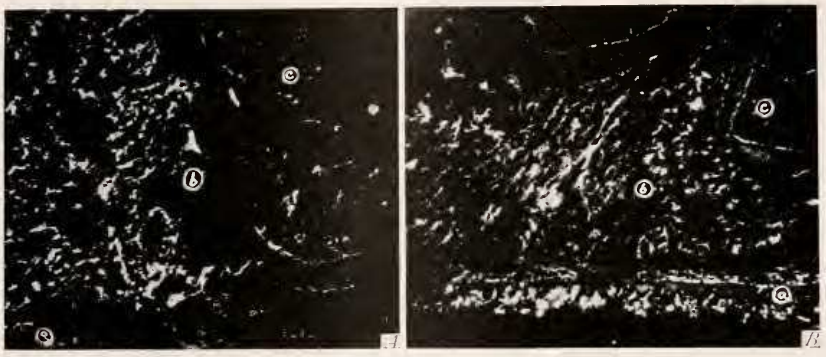

FIG. 1. Photomicrographs of incinerated preparations of *Phagocata*. Sections cut at 6 micra. (*A*) Young animal showing relatively large amounts of ash in the interstitial cells and small amounts of ash in the pharynges. (*B*) Old animal showing the increase in the ash content of the interstitial cells and the epithelial cells. Ash outline of some of the cells of the pharynges can be distinguished. Epithelium (*a*), Interstitial cells (*b*), Pharynx (*c*). Eastman "Contrast Process Panchromatic" film; 500-watt projection lamp; 15-second exposure, 200 magnification.

there was an increase in the total amount of ash with age. The basal membrane of the epithelial lining of the body in young animals is very thin and difficult to detect, but in old specimens the basal membrane is a glaring white line of ash. The increase in total ash content of the cell membranes of epithelial, interstitial (Fig. 1), and nerve cells is apparent. There appear to be no detectable changes in the total ash content of the rhabdites or the nuclei in the epithelial layer with age. This may be due to the fact that even young preparations are very rich in inorganics in these structures and an increase would be difficult to detect. The tissue layers of the pharynges, which as previously stated contain relatively little ash, show little or no increase with age in total ash content (Fig. 1).

Application of the chemical tests for calcium demonstrates that calcium increases with age in all the cells studied. The epithelial layer

is rich in calcium in young as well as old specimens, but there is a marked increase in the calcium content of the cell membranes of the epithelial cells and basal membrane with age. There is an increase in the calcium content of the nuclei and cell membranes of the cells of the pharynges with age, but the change here is far less than it is in the epithelial layer. Repeated tests for calcium in the cells of the pharynges confirm this slight change with age in the calcium content. The cilia of the lining of the pharynges are apparent in most of the preparations but no changes in their calcium content could be observed. The most marked increase in calcium content with age in the cells of the pharynges occurs in the cell membranes of the cells lining the lumina. A pronounced increase in the calcium content of the interstitial and nerve cells was observed. Calcium increases with age in the nuclei, cytoplasm, and particularly the cell membranes of these cells.

Examination of the untreated incinerated slides indicates that the epithelial and interstitial cells, and the rhabdites are richest in iron content. The epithelial and nerve cells show no increase in iron content with age. The interstitial cells reveal an inconsistent fluctuation in iron content with age. The cytoplasm of these cells, in some preparations, shows an increase in iron content with age while in other preparations there was no perceptible difference in the iron content of young and old specimens.

The picture obtained in the animals of medium size was very similar to that found in the group of large planaria. However, the concentration of calcium in the various cells of the large planaria was somewhat greater than that found in the cells of the medium group.

Euchlanis dilatata

Incinerated preparations of this rotifer manifest the same age changes that were observed in *Phagocata.* There was no significant difference in the amount of total ash in the cells of the one- and two-day-old specimens. However, the three-day-old rotifers contained much more ash than did the younger material, and the amount of total ash increased still further in the four-day-old material.

Tests with sodium alizarin sulfonate and the gypsum reaction showed that the increase in the calcium content of the rotifers paralleled the total ash increase. Thus, the first indication of an increase in calcium content was observed in the three-day-old material and a further increase was found in the four-day-old rotifers. Difficulty was experienced in identifying the skeletons of the various cells in this rotifer and only the cells of the brain, gut, and ovary showed clearly in the incinerated prep-

arations. This may be due to the fact that the animals contract somewhat during the fixation process. Tests indicated that calcium increased markedly in the nuclei, cytoplasm and cell membranes of these cells in the three- and four-day-old rotifers. The cell membranes of the one- and two-day-old rotifers showed as thin rings delicately tinted with a red color after they were stained with alizarin. The cell membranes of the three- and four-day-old rotifer material showed as heavy rings of ash which were stained a deep red with the alizarin.

The iron content of the cells of *Euchlanis* appeared to vary somewhat with age, but the iron changes were not as consistent as in *Phagocata*. There was a distinct increase in the iron content of some of the four-day-old specimens over the younger material. On the other hand, specimens were found in which the iron content was higher in the two- and three-day-old material than it was in the four-day-old material. The iron was found to vary most in the nuclei and cell membranes.

Ehrlich's Haematoxylin

It was earlier stated that control slides were prepared which were stained with Ehrlich's haematoxylin. Routine checks made with these slides on incinerated preparations revealed a striking relationship between the intensity of haematoxylin staining of the various cell types and the calcium distribution and content of these cells. In agreement with Scott (1933) it was found that structures rich in calcium as determined by microincineration invariably stained a dark purple with the haematoxylin, and structures with a low calcium content stained but lightly. Thus, the cells of the pharynges of *Phagocata* were delicately stained with haematoxylin in contrast to the other body tissues of *Phagocata* which stained heavily. As observed previously, the pharynges were very low in calcium content. The basal membrane of the epithelial layer of *Phagocata,* which analysis for calcium following microincineration proved to be rich in calcium, stained a dense purple with the haematoxylin. Similarly, the cell membranes of the epithelial and interstitial cells which were demonstrated to be rich in calcium also stained very heavily with the haematoxylin. A comparison of stained slides of young and old planarian and toad material revealed that the old material under the same staining conditions invariably stained more heavily than did the young material, and that the regions that stained more heavily corresponded to those regions which manifest an increase in calcium content with age. This relation between the intensity of staining with Ehrlich's haematoxylin and the calcium content has been frequently observed by the author in various types of material.

The use of haematoxylin as an indicator for calcium has been criticized by Lison (1936) in his excellent book on histochemical methods. He states that the color reaction between haematoxylin and calcium is dependent upon the presence of aluminum, chromium or gold in the fixatives used. However, despite the fact that the reaction is indirect, the close correlation between the calcium content of a structure and the intensity of haematoxylin staining of that structure justifies the use of this simple staining procedure for the identification and localization of calcium in cells and tissues.

Conclusions

The present studies demonstrate that a calcium increase with age, observed in the cortex of *Elodea* cells (Lansing, 1942), occurs similarly in the cells of two invertebrates and in the muscle tissue of a vertebrate. The widespread occurrence of this localized calcium increase with age suggests that it is a general characteristic of the aging process.

Further development of the relation between calcium and aging from both a descriptive and experimental point of view will require an objective quantitative method for the determination and localization of calcium in cells. Further, since it has been demonstrated that calcium increases with age in many tissues and organs of mammals (Lansing, 1942), it would be pertinent to determine whether the calcium increase with age in mammals also takes place in the cell membranes.

Summary

A rotifer, *Euchlanis dilatata,* a planarian, *Phagocata* sp., and toad, *Bufo fowleri,* were selected for an analysis of calcium localization and of possible calcium increase with age. Samples of animals of different ages were sectioned, incinerated and examined with a darkfield microscope. Calcium was identified by means of sodium alizarin sulfonate and the gypsum reaction. It was demonstrated that calcium increased with age in the cell membranes of all the cells studied. Calcium also increased with age in the nuclei and cytoplasm of nerve and interstitial cells of *Phagocata* and the cells of *Euchlanis.* Because of the large amounts of calcium present in the nuclei it was difficult to determine the degree of calcium increase.

The iron content of the various cells appeared to vary considerably but inconsistently with age.

Control slides stained with Ehrlich's haematoxylin showed a correlation between the intensity of staining of structures and the calcium content of those structures. The suggestion was made that Ehrlich's haematoxylin may be used as an indicator for bound calcium in cells.

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